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Journal

Epilepsy research, 14(2)

ISSN

0920-1211

Authors

Ribak, CE
Lauterborn, JC
Navetta, MS
et al.

Publication Date

1993-02-01

DOI

10.1016/0920-1211(93)90015-y

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Peer reviewed

EPIRES 00543

The inferior colliculus of GEPRs contains greater numbers of cells that express glutamate decarboxylase (GAD₆₇) mRNA

Charles E. Ribak, Julie C. Lauterborn, Marco S. Navetta and Christine M. Gall

Department of Anatomy and Neurobiology, University of California, Irvine, CA 92717, USA

(Received 23 June 1992; revision received 11 September 1992; accepted 27 October 1992)

Key words: GABAergic neurons; In situ hybridization; Autoradiography; Genetic epilepsy

Previous studies have shown significantly greater GABA levels and numbers of GABAergic neurons in the central nucleus of the inferior colliculus (ICCN) of genetically epilepsy-prone rats (GEPR-9s). In the present study, in situ hybridization and emulsion autoradiographic techniques were used to determine whether there are also elevated numbers of ICCN cells that contain the 67-kD form of mRNA for the GABA synthesizing enzyme, glutamate decarboxylase (GAD), in GEPR-9s as compared to normal Sprague-Dawley (SD) rats. Hybridization with a ³⁵S-labeled RNA probe complementary to a span of monkey GAD mRNA labeled cells throughout the brain including the ICCN. Labeled cells in the ICCN appeared to be of different sizes that corresponded with previous descriptions of GABAergic neurons from immunocytochemical studies. In the GEPR-9s, a larger number of GAD₆₇ cRNA labeled neurons was observed in the ICCN as compared to SD rats. The external nucleus of the inferior colliculus was also found to contain significantly greater numbers of GAD₆₇ cRNA labeled neurons whereas in the frontal cortex, a region of the brain that is not required for audiogenic seizure activity in GEPR-9s, there were no significant differences in hybridization between GEPR-9s and SD rats. Interestingly, within the superficial layers of the superior colliculus there was a higher density of hybridization in GEPR-9s than in SD rats indicating higher levels of GAD expression. The data on hybridization within the inferior colliculus are consistent with previous results that showed elevated numbers of GABAergic neurons in the central and external nuclei of GEPR-9s and provide further support for an abnormality of the GABAergic system in this model of genetic epilepsy.

Introduction

Previous studies have shown that γ -aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the inferior colliculus (IC) and plays a role in seizure initiation following auditory stimulation in both normal Sprague-Dawley (SD) rats and genetically epilepsy-prone rats (GEPR-9s)^{1,7,10}. For example, the GABA antagonist, bicuculline, has

been shown to induce audiogenic seizure-like activity in rats when injected into the IC¹⁰. In addition, morphological studies using immunocytochemistry with an antiserum to glutamate decarboxylase (GAD) have shown greater numbers of GABAergic and total neurons in the IC of GEPR-9s as compared to non-epileptic SD rats^{15,16}. Furthermore, biochemical analysis of the central nucleus of the IC has revealed elevated GABA content in the GEPR-9s as compared to SD rats¹³. Thus, there appears to be an abnormality of the GABAergic system in the IC of GEPR-9s, and it may play a vital role in their seizure susceptibility. The objective of this study was to use in situ hybridiza-

Correspondence to: Dr. Charles E. Ribak, Department of Anatomy and Neurobiology, University of California, Irvine, CA 92717, USA.
Tel.: (714) 856-5494.

tion to determine whether abnormalities in the numbers and distributions of neurons containing GAD₆₇ mRNA exist in the inferior colliculus of GEPR-9s.

In the present study, we examined the autoradiographic localization of hybridization of a ³⁵S-labeled RNA probe, complementary to the 67-kD form of GAD mRNA, in tissue sections through the three major nuclei of the IC (the central nucleus, external nucleus and dorsal cortex) of both GEPR-9s and SD rats. Light microscopy was used to count the labeled neuronal cell bodies. Also, counts were made in the frontal cortex, an area where the number of labeled neuronal cell bodies in the GEPR-9s and SD rats was not anticipated to differ because this region is not required for the initiation or propagation of seizures in GEPR-9s⁴ and previous biochemical studies showed no differences in GABA content¹³. This analysis has shown that the number of cells that express GAD₆₇ mRNA is greater in the central and external nuclei of the IC of GEPR-9s.

Methods

Genetically epilepsy-prone rats that exhibited maximal seizures (GEPR-9s) (n=3) and age matched Sprague-Dawley (SD) rats (n=3) were used in the present study. Paired GEPR-9s (without evidence of seizure behavior immediately prior to sacrifice) and SD rats were killed by sodium pentobarbital overdose and perfused intracardially with 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2 (PB). The brains were postfixed in the perfusate for 24 h at 4°C and then transferred to 20% sucrose in 4% paraformaldehyde/PB for 48 h at 4°C. Sections of 30 μm thick were cut in the sagittal plane using a freezing microtome and collected into cold 4% paraformaldehyde/PB and stored at 4°C until processed.

Tissue sections were processed for the in situ hybridization localization of glutamate decarboxylase (GAD₆₇) mRNA as previously described⁹. Briefly, sections were pretreated with proteinase K (1 μg/ml in 0.1 M Tris buffer, pH 8.0, with 50 mM EDTA) at 37°C for 30 min, and then were incubated with a ³⁵S-labeled cRNA prepared from cDNA

pBSGAD^{2,3} (provided by D.L. Benson and E.G. Jones). The GAD cDNA corresponds to bases 1324–1683 of cat GAD cDNA reported to encode the 67-kD form of GAD^{2,6}. The antisense cRNA was transcribed from the *Bam*HI linearized cDNA using T7 RNA polymerase in the presence of ³⁵S-UTP. The incubation with the labeled cRNA was done in a hybridization buffer containing 50% formamide, 10% dextran sulfate, 0.7% Ficoll, 0.7% polyvinyl pyrrolidone, 0.7% bovine serum albumin, 0.15 mg yeast tRNA, 0.33 mg/ml denatured herring sperm DNA, 40 μM dithiothreitol, 24 mM sodium chloride and 2.4 mM sodium citrate. The hybridization incubation was done at 60°C for 30–40 h with the cRNA at 1 × 10⁶ cpm/100 μl. The sections were then treated with ribonuclease A (20 μg/ml in 10 mM Tris-saline, pH 8.0, with 1 mM EDTA) for 30 min at 45°C and washed through descending concentrations of saline sodium citrate (SSC) buffer (pH 7.0) to a final stringency of 0.1 × SSC. The sections were mounted onto gelatin-coated-slides and air-dried.

The distribution of cellular cRNA hybridization was evaluated using emulsion autoradiography (Kodak NTB2 nuclear track emulsion mixed 1:1 with H₂O) and exposures of 2–3 weeks at 4°C. Following the development of the tissue autoradiograms, the sections were stained with cresyl violet and coverslipped with Permount.

Controls for the cRNA probe hybridization specificity included hybridization of tissue with the ³⁵S-labeled sense RNA sequence and treatment of tissue sections with ribonuclease A prior to normal incubation with the antisense cRNA probe. The sense-strand RNA was prepared by transcription from *Pvu*II linearized pBSGAD using T3 RNA polymerase in the presence of ³⁵S-UTP. In both cases, hybridization was not detected. In addition, the distribution of the hybridization-labeled neurons in the present material agreed with previous immunocytochemical reports on the localization of GAD-containing cells in the rat brain, including the inferior colliculus^{11,16}.

The labeled neuronal cell bodies were counted using a light microscope at 20 × objective magnification. Dark-field microscopy was used to visualize the neuronal cell bodies that were labeled by the autoradiographic method. If a cluster of silver

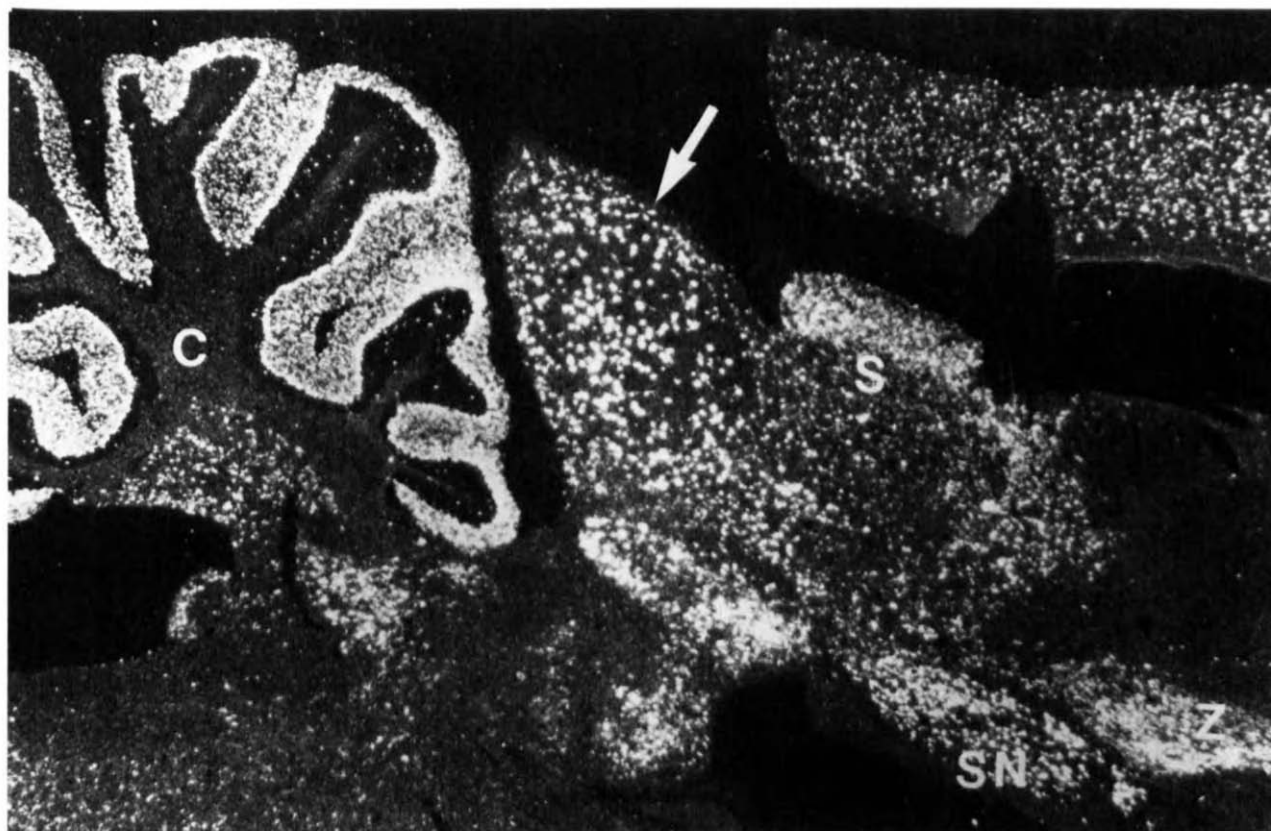


Fig. 1. Low magnification darkfield photomicrograph of a parasagittal section through the brain of a SD rat. The section shows numerous GAD₆₇ cRNA labeled cells in many brain regions, including the cerebellum (C), inferior colliculus (arrow), superior colliculus (S), substantia nigra (SN) and zona incerta (Z). $\times 15$.

grains was observed over a cell body and the cluster displayed a significantly greater number of grains than the background (by two standard deviations), this neuron was determined to be autoradiographically labeled by the *in situ* hybridization method. Labeled cells in both GEPR-9s and SD rats were counted on coded slides by the same individual.

Counts were made for the central nucleus of the IC and the frontal cortex using a grid with an area of $32,250 \mu\text{m}^2$. A similar method was used for counting the external nucleus and dorsal cortex of the IC except that only half of the grid was used. In this way, it was possible to determine specifically the number of labeled cells in the latter two regions and to eliminate the possible error of counting adjacent regions of the IC (e.g., the central nucleus). Each area was identified using previously described criteria^{8,12}.

The Macintosh StatView program was used to

obtain the means and standard errors for the GEPR-9s and SD rats. Statistical significance was determined with an unpaired *t*-test.

Results

The *in situ* hybridization autoradiograms of parasagittal sections through the brains of GEPR-9s and SD rats showed specific hybridization of the GAD₆₇ cRNA to neurons throughout many brain regions (Fig. 1). The distribution of labeled neurons was similar to that previously described for this cRNA². For example, Purkinje cells were densely labeled in the cerebellar cortex. Other autoradiographically labeled neurons were found in the inferior colliculus, cerebral cortex, superior colliculus, zona incerta, thalamic reticular nucleus, substantia nigra and throughout the brainstem. The appearance and number of labeled cells were most evident using darkfield illumination (Fig. 1). How-

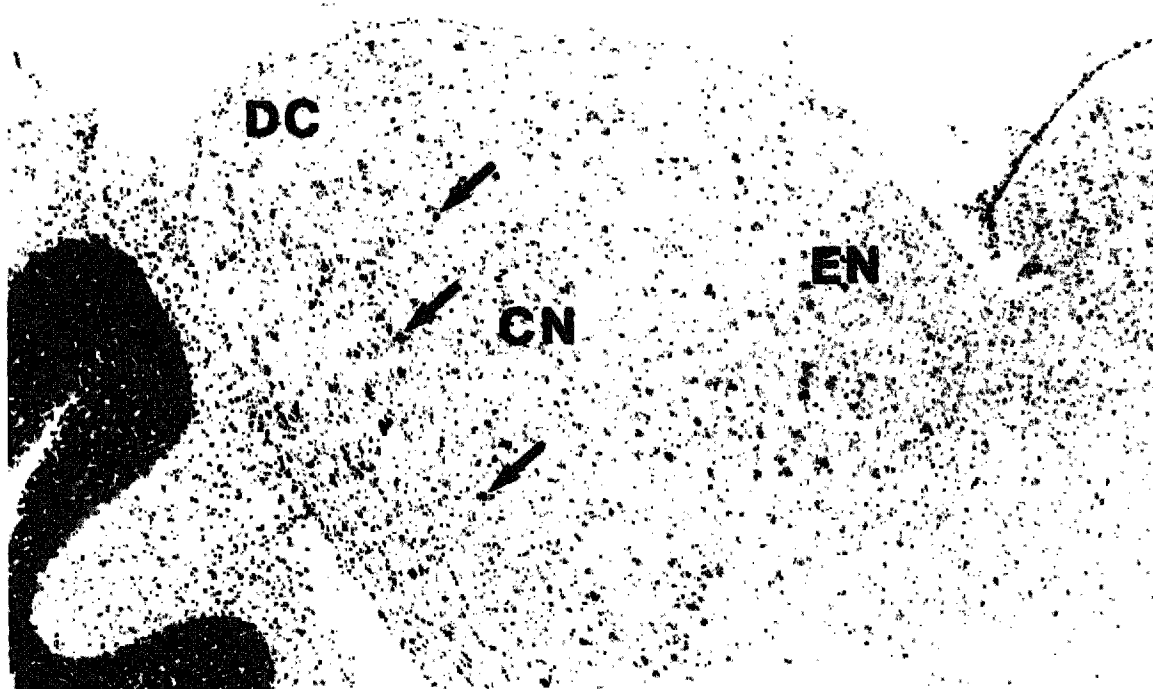


Fig. 2. Brightfield photomicrograph of the inferior colliculus from a GEPR-9. Note that despite the light Nissl staining many large cells show dense accumulations of silver grains (arrows). Boundaries between the various subnuclei of the inferior colliculus are approximated by the following abbreviations: central nucleus, CN; external nucleus, EN; dorsal cortex, DC. $\times 75$.

ever, it is important to note that in brightfield optics many large, Nissl stained cell bodies were overlaid with clusters of black silver grains (Fig. 2). Therefore, the clusters of grains observed in dark-field optics corresponded to neuronal cell bodies and were not found to be overlying capillaries or glial cells.

The inferior colliculus (IC) of the GEPR-9s (Fig. 3B) contained more autoradiographically labeled cells than that of the SD rats (Fig. 3A). This increase in number was observed to be greatest in the central nucleus of the IC. Within this region there was a broad range in the apparent size of the cRNA labeled cells in the SD rat (Fig. 4). A similar range of sizes for cRNA labeled cells was found in GEPR-9s but the number of labeled small cells (with nuclei less than $15\ \mu\text{m}$ in diameter) appeared to be greater in these rats (Fig. 4B). The quantitative results confirmed these qualitative observations (Fig. 5). In the central nucleus of the IC the mean number of labeled cells per unit area was 67.7 in GEPR-9s as compared to 37.5 in SD rats. Quantification of GAD_{67} cRNA labeled

cells in the external nucleus of the IC indicated a difference in this region as well. Specifically, GEPR-9s had 64 labeled cells per unit area compared to 38.5 for SD rats. The means for both of these nuclear regions were significantly different ($P < 0.01$, unpaired *t*-test). Cell counts from the dorsal cortex of the IC and the frontal cortex revealed no significant differences in GAD_{67} cRNA labeling between GEPR-9s and SD rats (Fig. 5), although the dorsal cortex of the IC showed a trend for greater numbers of cells in GEPR-9s.

The number of silver grains overlying similar-sized, labeled neuronal somata in the IC of GEPR-9s appeared to be equivalent to that of SD rats. In contrast, in the superficial layers of the adjacent superior colliculus, individual neurons appeared to be labeled with a greater density of autoradiographic grains in the GEPR-9s as compared to the SD rats. However, the elevated labeling density in the superior colliculus did not appear to be associated with an increase in the number of labeled neurons (Fig. 3A,B).

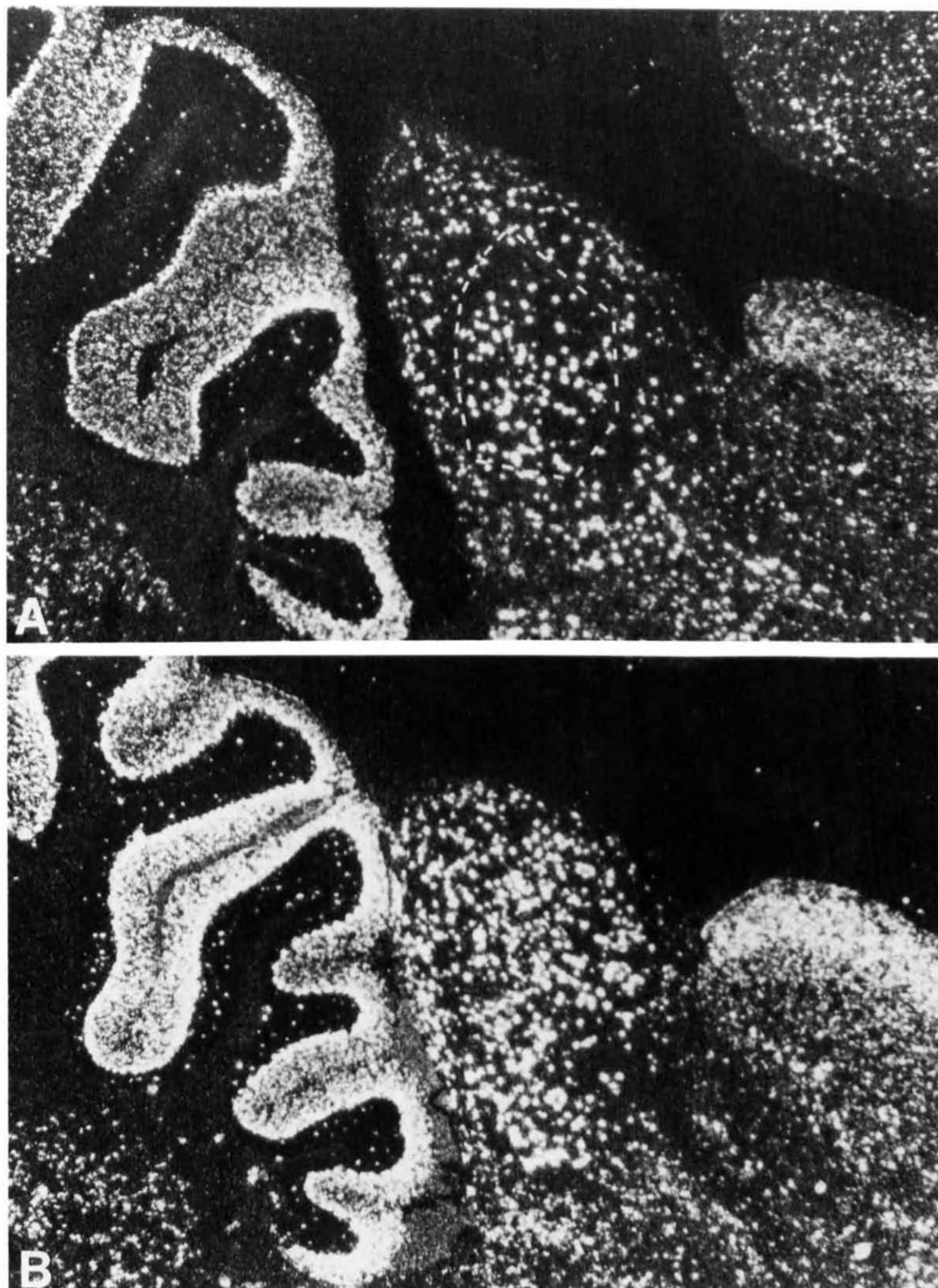


Fig. 3. Darkfield photomicrographs of areas shown in Figs. 1 and 2 for the SD rat (A) and GEPR-9 (B), respectively. Note the elevated number of labeled neurons in the inferior colliculus from the GEPR-9 (B) as compared to that of the SD rat (A). The boundary of the central nucleus of the inferior colliculus is indicated by a white broken line in A. The superior colliculus shows an increase in the density of labeling but not in the number of labeled cells. $\times 50$.

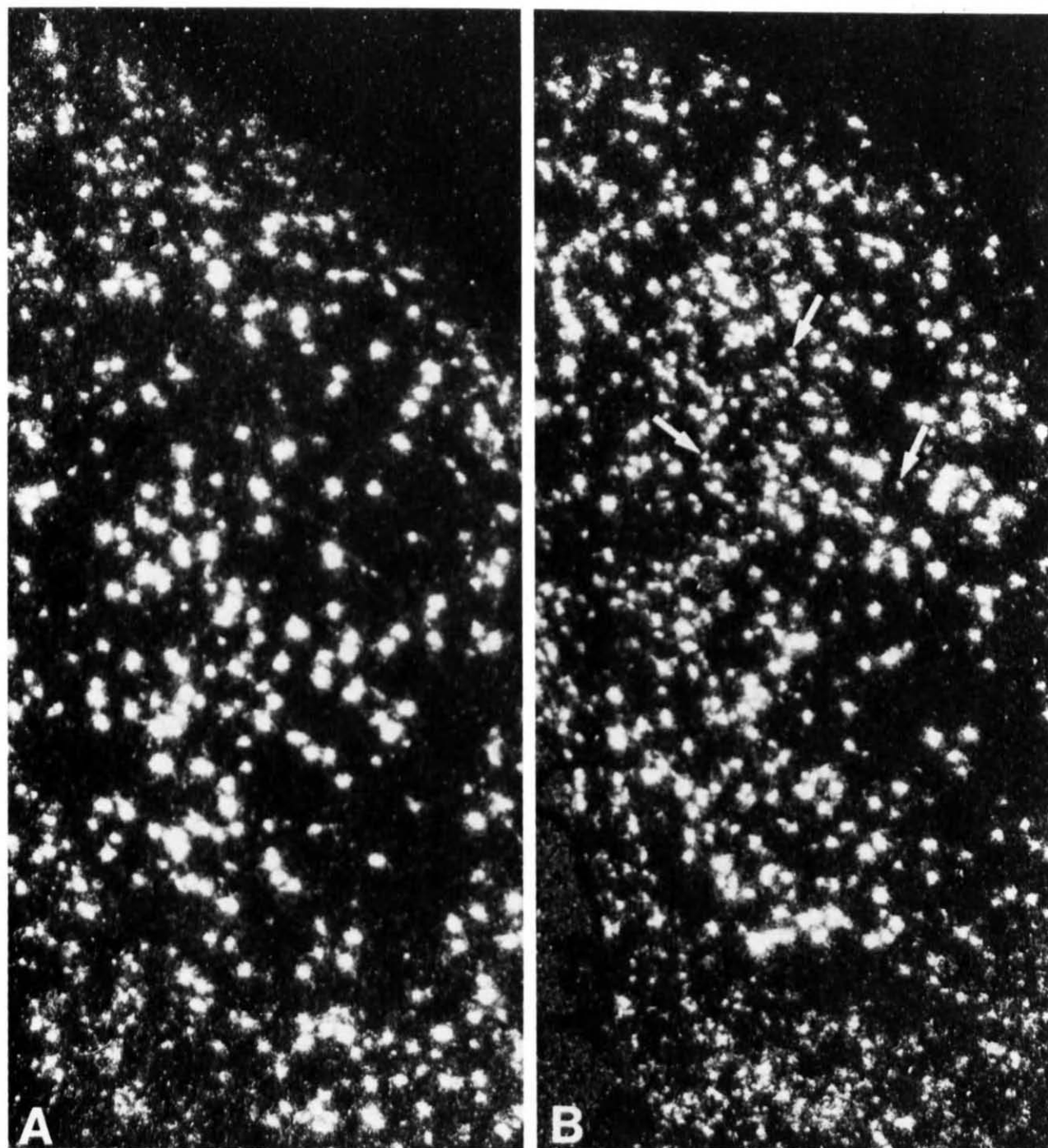


Fig. 4. Enlargements of the central nucleus of the IC from Fig. 3A,B to show that the increase in the number of labeled cells in the GEPR-9 (B) as compared to the SD rat (A) is due primarily to an increase in the number of small neurons (arrows in B). This finding is consistent with previous immunocytochemical data that also showed an increase in the number of small GABAergic neurons in the central nucleus of the IC of GEPR-9s. $\times 160$.

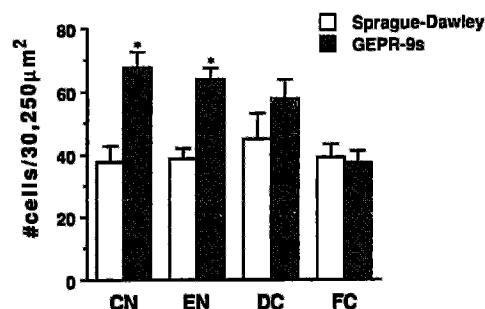


Fig. 5. Histogram of the quantitative data to show the mean number of labeled neurons that express GAD_{67} mRNA in the three regions of the inferior colliculus, the central nucleus (CN), external nucleus (EN) and dorsal cortex (DC). These data were obtained from three SD rats that did not display audiogenic seizures and from three GEPR-9s that displayed maximal audiogenic seizures. The frontal cortex (FC) was also counted. Asterisks indicate significant differences in GEPR-9s as compared to SD rats. Standard errors are indicated by small brackets.

Discussion

The major finding of this study is that the number of neurons that express GAD_{67} mRNA is significantly greater in the central and external nuclei of the inferior colliculus (IC) of GEPR-9s as compared to SD rats. These data confirm the previous immunocytochemical and biochemical data that showed greater numbers of GAD-immunoreactive neurons and GABA content, respectively, in GEPR-9s^{13,16}. The results of these studies support the hypothesis that an abnormality of the GABAergic system exists in the inferior colliculus of this genetic model of epilepsy.

The greater number of GAD_{67} cRNA labeled cells in the IC of GEPR-9s observed in the present study probably reflects an elevated number of GAD-containing cells as previously reported by Roberts et al.¹⁶ rather than aberrant GAD_{67} mRNA expression by populations of normally non-GABAergic neurons of the IC. Previous quantitative studies of 40 μ m thick and 2 μ m semithin sections showed that there were more neurons in the central nucleus of the IC of GEPR-9s as compared to SD rats^{15,16}, and that this increase was due to a greater number of small neurons. The category of small cells was also found to be greater in the quantitative study of GAD-positive neurons in GEPR-9s¹⁶. Similarly, in the present study we observed elevated numbers of small GAD_{67}

cRNA labeled cells in the IC of GEPR-9s.

There was no apparent difference in the density of autoradiographic grains overlying GAD_{67} cRNA labeled cells in the IC from SD rats and GEPR-9s (cf. Fig. 4A,B). Thus, GAD_{67} mRNA content does not appear to be expressed at supra-normal levels by IC neurons in GEPR-9s. However, we cannot exclude the possibility that a subpopulation of neurons that express GAD_{67} mRNA at very low levels and are not detectably labeled with the cRNA in SD rats increase expression to detectable levels in GEPR-9s, and are therefore only counted as GAD_{67} mRNA-positive in this latter group. It should be noted that this possibility is also relevant to immunocytochemical studies where greater numbers of GAD-immunoreactive neurons were counted. However, the greater total number of neurons in the IC of GEPR-9s, as compared to SD rats¹⁶, suggests that differences between numbers of GAD-synthesizing neurons contribute to the increased counts found in both in situ hybridization and immunocytochemical preparations. Moreover, the immunocytochemical study showed that the proportion of GAD-positive neurons to total neurons was greater in GEPR-9s as compared to SD rats¹⁶. Since the ribonuclease treatment included in the present in situ hybridization method removes much of the Nissl staining capacity of neurons, it is not possible to count with confidence the numbers of total neurons in such preparations and determine whether the proportion of GAD_{67} mRNA labeled cells is also greater in GEPR-9s.

In contrast to this finding in the IC, the superficial layers of the superior colliculus of GEPR-9s showed a higher density of silver grains overlying individual neurons. This finding indicates that GABA is synthesized in greater amounts in GEPR-9s in this region of the mesencephalon. The superior colliculus is considered an important structure for the propagation of seizures in this model of epilepsy¹⁴. Thus, the superior colliculus provides further evidence for a link between aberrations in GAD_{67} mRNA expression and seizure propagation. It will be of interest to determine if GABA mediated inhibition, as assessed electrophysiologically, is abnormal in the superior colliculus as has been reported for the inferior colliculus

of GEPR-9s⁷.

Although the data support the existence of a defect in GAD expression in the IC of GEPR-9s, it is probably not the only abnormality that is underlying epileptogenesis in the IC of this model. Previous biochemical studies have shown not only elevated GABA content in the IC of GEPR-9s but also greater than normal levels of the excitatory neurotransmitter, glutamate¹³. Therefore, both inhibitory and excitatory mechanisms appear to be abnormal in GEPR-9s.

Finally, the data from the present study confirm that a change in the number of GABAergic neurons occurs in the IC of GEPR-9s. The elevated number of GABAergic neurons is indicative of a greater potential for GABA release and action. However, there are two forms of GAD mRNA, 65 kD and 67 kD (also known as GAD₆₅ and GAD₆₇, respectively) that are encoded by different genes^{5,6} and are differentially regulated in neurons of adult brains under some experimental conditions. For example, levels of GAD₆₇ mRNA, the

form evaluated in the present study, are altered within the striatum following deafferentation¹⁷. It will be of interest to determine if GAD₆₅ mRNA is also abnormal and elevated in IC of GEPR-9s and if the expression of these mRNAs is abnormal prior to the developmental appearance of seizure activity. Abnormalities in the expression of both isoforms in neonatal GEPR-9s would argue for congenital disturbances in the regulation of GAD gene expression in this model of epilepsy as opposed to alterations in GAD expression that are secondary to changes in physiological activity (i.e., seizures) or connectivity.

Acknowledgements

The authors gratefully acknowledge Drs. Deanna Benson and Edward G. Jones for providing the GAD₆₇ cDNA (supported by NIH Grants NS 21377 and EY 07193). This work was supported by NIH Grants NS 15669 (C.E.R.) and NS 26748 (C.M.G.).

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